



Lycopene suppresses ovalbumin-induced airway inflammation in a murine model of asthma

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ABSTRACT

In this study, we attempt to determine whether lycopene regulates inflammatory mediators in the ovalbumin (OVA)-induced murine asthma model. To address this, mice were sensitized and challenged with OVA, and then treated with lycopene before the last OVA challenge. Administration of lycopene significantly alleviated the OVA-induced airway hyperresponsiveness to inhaled methacholine. Administration of lycopene also resulted in a significant inhibition of the infiltration of inflammatory immunocytes into the bronchoalveolar lavage, and attenuated the gelatinolytic activity of matrix metalloproteinase-9 and the expression of eosinophil peroxidase. Additionally, lycopene reduced the increased levels of GATA-3 mRNA level and IL-4 expression in OVA-challenged mice. However, it increased T-bet mRNA level and IFN- γ expression in lycopene-challenged mice. These findings provide new insight into the immunopharmacological role of lycopene in terms of its effects in a murine model of asthma.

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Asthma is a chronic inflammatory lung disease that is characterized by airway hyperresponsiveness (AHR) to allergens, airway edema, and increased mucus secretion [1,2]. Inflammation occurs due to the infiltration of eosinophils, neutrophils, macrophages, and lymphocytes into the bronchial lumen and lung tissues [3,4]. Recruitment of these inflammatory cells from the blood to the site of inflammation is regarded as a critical event in the development and persistence of airway inflammation [5,6]. In particular, it has been reported that the infiltration of eosinophils into the asthmatic lung leads to degranulation and the release of eosinophil peroxidase (EPO), resulting in airway epithelial damage and the development of AHR [7,8]. Recent reports have shown that infiltrated eosinophils in the lung preferentially stimulate T-helper type 2 (Th2) cell responses by presenting antigens [9–11]. Therefore, Th2 cells are dominant in the airways [12] and Th2 cytokines such as IL-4, IL-5, and IL-13 play a pivotal role in the pathophysiology of asthma [13–15]. Matrix metalloproteinase-9 (MMP-9) also appears to induce the migration of eosinophils, lymphocytes, neutrophils, and dendritic cells across basement membranes during tissue injury and repair [16,17]. The recruitment of leukocytes from the cir-

culating blood into the lung has been shown to play an especially important role in the induction of airway inflammation through inflammatory mediators.

Lycopene is the red pigment in tomatoes and is known as a potent anti-oxidant [18,19]. Dietary intake of tomatoes and tomato products is reportedly associated with a decreased risk of cardiovascular disease and several types of cancer, especially prostate cancer [20–22]. We also reported in a previous study that lycopene exhibited profound immunoregulatory activities both *in vitro* and *in vivo*, particularly in dendritic cells [23].

In this study, our findings demonstrated, for the first time, that lycopene treatment inhibits the asthmatic syndrome through blocking the infiltration of inflammatory cells and suppressing inflammatory mediators such as the gelatinolytic activity of MMP-9, and expression of EPO and IL-4 in airway inflammation.

Materials and methods

Animals and experimental protocol. Female BALB/c mice, 6–8 weeks of age and free of murine-specific pathogens, were obtained from the Charles River Laboratories (Yokohama, Japan). All experimental animals used in this study were maintained under a protocol approved by the Institutional Animal Care and Use Committee of the Pusan National University Medical School. Mice were

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immunized intraperitoneally (i.p.) with 20 µg of OVA (Sigma; St. Louis, MO) emulsified in 1 mg of aluminum hydroxide (Pierce; Rockford, IL) on days 1 and 15. Mice were challenged for 30 min via the airway with OVA (50 mg/ml of saline) each day from days 21–23 on consecutive days. Bronchoalveolar lavage (BAL) fluid was obtained at 48 h after the last challenge. At the time of lavage, the mice (5 mice in each group) were euthanized with an overdose of ether. The chest cavity was exposed to allow for expansion, after which the trachea was carefully intubated and the catheter secured with ligatures. Prewarmed saline solution was slowly infused into the lungs and withdrawn. The aliquots were pooled and then kept at 4 °C. A part of each pool was then centrifuged, and the supernatants were kept at –70 °C until use.

Administration of lycopene. Mice were injected i.p. with 8 or 16 mg/kg/day in 200 µl of lycopene (Sigma) each day from days 18–20 on consecutive days.

Total cell counting. The total cell numbers were counted with a hemacytometer. Smears of BAL cells prepared with Cytospin II (Shandon; Runcorn, UK) were stained with Diff-Quik solution (Dade Diagnostics; Aguada, PR) for differential cell counting. Two independent, blinded investigators counted the cells using a microscope. Approximately 200 cells were counted in each of four different random locations.

Measurement of EPO. The suspension of BAL cells and the pulmonary homogenates were frozen and thawed three times using liquid nitrogen and a water bath at 37 °C to obtain the EPO. The BAL fluid (BALF) was centrifuged at 4 °C for 10 min and serially diluted in a 96-well plate (75 µl/well); this was followed by the addition of 150 µl of substrate (1.5 mM *o*-phenylenediamine and 6.6 mM H₂O₂ in 0.05 M Tris–HCl [pH 8.0]). After 30 min at room temperature, the reaction was stopped by the addition of 75 µl of 30% H₂SO₄, and the absorbance of the samples was determined to be 492 nm in ELISA reader.

Histology. At 48 h after the last challenge, the lungs were removed from the mice after they had been sacrificed. Prior to the removal of the lungs, the lungs and trachea were filled intratracheally with a fixative (4% paraformaldehyde) using a ligature around the trachea. Lung tissues were fixed with 10% (v/v) paraformaldehyde. The specimens were dehydrated and embedded in paraffin. For histological examination, 4 µm sections of fixed embedded tissues were cut on a Leica model 2165 rotary microtome (Leica; Nussloch, Germany), placed on glass slides, deparaffinized, and sequentially stained with hematoxylin 2 and eosin-Y (Richard-Allan Scientific; Kalamazoo, MI).

RNA preparation and quantitative RT-PCR. The total RNA from lung tissues was isolated with the use of TRIzol (Invitrogen; Carlsbad, CA). Real-time PCR was performed on cDNA samples using the SYBR Green system (Bio-Rad; Richmond, CA). Primers used were MMP-9 sense 5'-GCCCTGGAACCTCACACGACA-3', MMP-9 antisense 5'-TTGGAAACTCACACGCCAGAAG-3'; T-bet sense 5'-CAA CAA CCC CTT TGC CAA AG-3', T-bet antisense 5'-TCC CCC AAG CAG TTG ACA GT-3'; and GATA-3 sense 5'-GAG GTG GAC GTA CTT TTT AAC ATC G-3', GATA-3 antisense 5'-GGC ATA CCT GGC TCC CGT-3'. Cycling conditions were 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min and 40 cycles each corresponding to 15 s at 95 °C and 1 min at 60 °C. Analysis was performed using the sequence detection software supplied with the instrument. The relative quantitation value is expressed as 2 ± DCT, where DCT is the difference between the mean CT value of duplicates of the sample and of the GAPDH control.

Measurement of cytokines. Levels of IL-4 and IFN-γ were quantified in BALF by enzyme immunoassays performed according to the manufacturer's protocol (R&D Systems; Minneapolis, MN).

Determination of airway responsiveness to methacholine. Airway responsiveness was measured in mice 24 h after the last challenge in an unrestrained conscious state, as described previously [24].

Mice were placed in a barometric plethysmographic chamber (All Medicus Co., Seoul, South Korea) and baseline readings were taken and averaged for 3 min. Aerosolized methacholine in increasing concentrations (2.5 to 50 mg/ml) was nebulized through an inlet of the main chamber for 3 min. Readings were taken and averaged for 3 min after each nebulization. Enhanced pause (Penh), calculated as (expiratory time/relaxation time–1) × (peak expiratory flow/peak inspiratory flow), according to protocol of the manufacturers, is a dimensionless value that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and a function of the timing of expiration. Penh was used as a measure of airway responsiveness to methacholine. Results were expressed as the percent increase of Penh following challenge with each concentration of methacholine, where the baseline Penh (after saline challenge) was expressed as 100%. Penh values were averaged for 3 min after each nebulization and evaluated.

Zymography. Gelatin zymography was used to assess the activity of MMP-9. Briefly, 10 µl of BALF was subjected to electrophoresis in a 10% polyacrylamide gel containing 1% gelatin (Bio-Rad). The gel was incubated in a renaturing buffer (2.5% Triton X-100 buffer) for 30 min to remove the sodium dodecyl sulfate. After rinsing, the gels were incubated (37 °C for 20 h) in an enzyme activation buffer (50 mM Tris–HCl [pH 7.3], 200 mM NaCl, and 0.02% Tween 20). The gels were then stained with Coomassie brilliant blue R250 stain and destained (5% methanol and 7% acetic acid in PBS solution), and the gelatinolytic activity was analyzed as clear bands.

Statistics. Experiments were repeated at least three times with consistent results. Unless otherwise stated, data are expressed as the mean ± SEM. ANOVA was used to compare experimental to control values while comparisons between multiple groups were performed using Tukey's Multiple Comparison test. Statistical significance was indicated by a *p* value of ***p* < 0.01 and **p* < 0.05.

Results

Lycopene suppresses the infiltration of inflammatory cells into the lung

Histological analyses revealed typical pathologic features of asthma in OVA-challenged mice showing numerous inflammatory cells around the bronchioles. As shown in Fig. 1A, mice treated with lycopene showed significant reductions in the infiltration of inflammatory cells in the peribronchiolar and perivascular regions. In particular, the number of neutrophils, eosinophils, lymphocytes, and macrophages in BAL was increased compared to the control group at 48 h after the OVA challenge. The administration of lycopene significantly increased neutrophils, eosinophils, lymphocytes, and macrophages in the airway lumen 2 days after last OVA sensitization (Fig. 1B). These results suggest that lycopene inhibits the influx of inflammatory cells into the lungs in OVA-induced airway inflammation.

Lycopene decreases airway hyperresponsiveness in OVA-induced murine asthma

Airway responsiveness was assessed as the percent increase of Penh in response to increasing doses of methacholine. In OVA-challenged mice, the dose–response curve of percent Penh was shifted to the left compared with that of control mice (Fig. 2). In addition, the percent Penh produced by methacholine administration (at doses from 2.5 to 50 mg/ml) increased significantly in the OVA-challenged mice compared with the controls. OVA-challenged mice treated with lycopene showed a dose–response curve of percent Penh that shifted to the right compared with that of untreated mice. These results indicate that lycopene treatment reduces OVA-induced airway hyperresponsiveness.

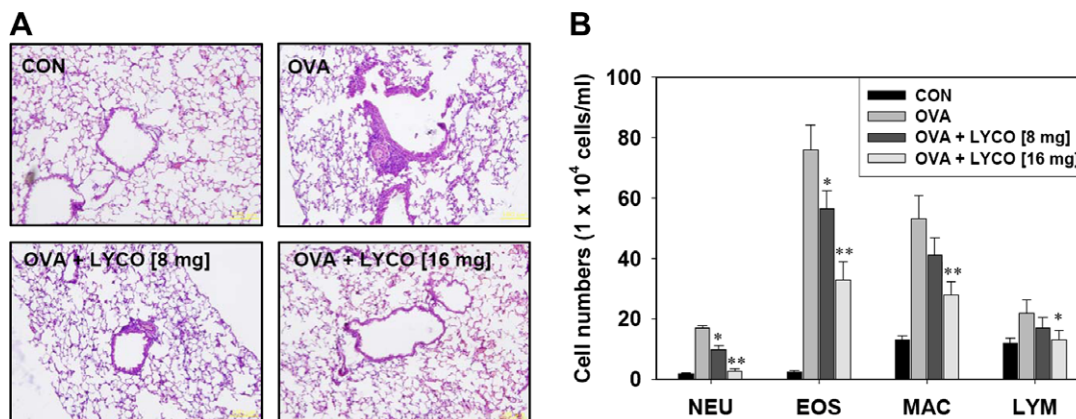


Fig. 1. Effect of lycopene (LYCO) on the infiltration of inflammatory cells. Mice were sensitized and challenged by OVA as described in the Materials and methods. (A) Lungs were removed 48 h after the last OVA-challenge. Sections were stained by haematoxylin and eosin staining (200 $\times$). (B) BAL cells were separated using a cytospin, and then stained with Diff-Quik. Differential cell counting was performed using standard morphological criteria. This experiment used 5 mice per group ($n=5$). * $p < 0.05$ and ** $p < 0.01$ vs. OVA-challenged mice. NEU, neutrophil; EOS, eosinophil; LYM, lymphocyte; and MAC, macrophages.

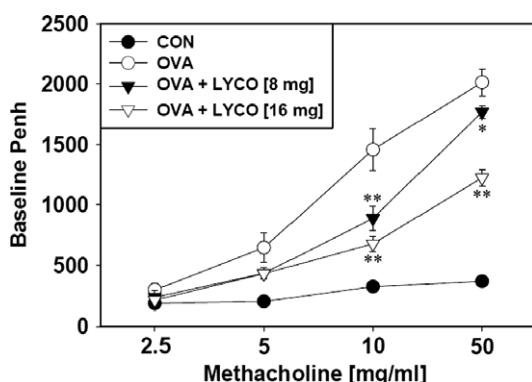


Fig. 2. Effect of lycopene (LYCO) on airway responsiveness in OVA-challenged mice. Mice were sensitized and challenged by OVA as described in the Materials and methods. Airway responsiveness to aerosolized methacholine was measured in unrestrained, conscious mice. Mice were placed into the main chamber and were nebulized first with PBS, then with increasing doses (2.5 to 50 mg/ml) of methacholine for 3 min for each nebulization. Data represent the means $\pm$ SEM from five independent experiments. * $p < 0.05$ and ** $p < 0.01$ vs. OVA-challenged mice.

Lycopene inhibits MMP-9 mRNA expression and gelatinolytic activity in BAL fluids

Because MMP-9 is known to be necessary for cell infiltration and invasion [16,17], we investigated whether lycopene inhibits MMP-9 mRNA expression and gelatinolytic activity in OVA-challenged mice. As shown in Fig. 3A, OVA-challenged mice had a 2.5-fold increase in the level of MMP-9 mRNA over that of control mice. Furthermore, OVA-challenge in mice also induced a marked induction of MMP-9 activity in BAL fluids in comparison to control mice. When 8 mg/kg/day lycopene was administered, the increased MMP-9 gelatinolytic activity was significantly inhibited (Fig. 3B). These results indicate that lycopene regulates MMP-9 gelatinolytic activity in lung tissues.

Lycopene decreases the level of EPO in BAL fluids

Because EPO has been reported to be significantly increased in OVA-induced asthma, we addressed whether lycopene decreased EPO expression in the lung. As shown in Fig. 3C, the expression of EPO was increased at 24 h after OVA inhalation compared with the levels after saline inhalation. However, this increased expres-

sion of EPO was significantly reduced by about 68.8% (8 mg) and 73.4% (16 mg) upon administration of lycopene. These results are related to attenuation of eosinophil infiltration and activation by administration of lycopene.

Lycopene attenuates GATA-3 mRNA and IL-4 expression in BAL fluids

Because asthma is favor to Th2-dominant immune reaction, we investigated the expression of the Th1 transcription factor, T-bet, and the Th2 transcription factor, GATA-3. Real-time PCR analysis revealed that expression of GATA-3 mRNA, but not T-bet, in lung tissues was significantly increased at 24 h after OVA-challenge compared with the levels after saline-challenge (Fig. 4A). The increased mRNA expression of GATA-3 was significantly decreased by the administration of lycopene. Conversely, treatment with lycopene resulted in an increase of T-bet. Additionally, BAL fluids were obtained 4 h after the last OVA-challenge and the expression of cytokines, IL-4 and IFN- γ , was analyzed by ELISA. As shown in Fig. 4B, the levels of IL-4 in the BAL fluids were significantly increased by airway challenge with OVA when compared with those of the control, but the level of Th1 cytokine, IFN- γ , was not changed as compared to saline-challenged mice. These results indicate that lycopene treatment inhibits Th2 cytokine levels in OVA-induced airway inflammation.

Discussion

This study is the first to provide experimental evidence demonstrating that lycopene inhibits OVA-induced airway inflammation in a murine model of asthma. Administration of lycopene profoundly inhibited asthmatic reactions such as leukocyte recruitment into the lung. Lycopene also suppressed the expression of inflammatory mediators such as MMP-9 and EPO. We also demonstrated that lycopene regulated the Th1/Th2 balance to suppress OVA-induced airway inflammation.

The MMPs are a family of zinc- and calcium-dependent endopeptidases capable of proteolytically degrading many of the components of the extracellular matrix [25]. One of the MMPs, MMP-9 can significantly induce the migration of eosinophils, macrophages, lymphocytes, and dendritic cells into inflammatory sites [26–28]. Moreover, the administration of an MMP-9 inhibitor reduces the migration of inflammatory cells through the endothelial and epithelial basement membranes [29]. Therefore, inhibitors of MMP-9 are good targets for anti-inflammatory effects in general and also in asthma. In our model, we evaluated

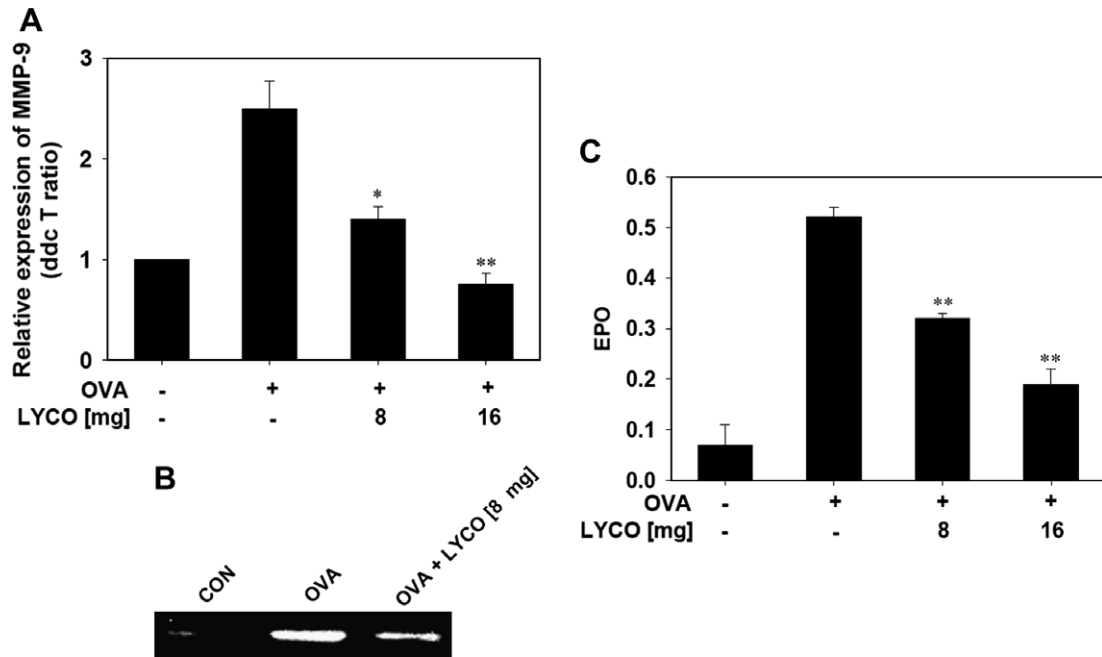


Fig. 3. Effect of lycopene (LYCO) on MMP-9 and EPO expression. Mice were sensitized and challenged by OVA as described in the Materials and methods. (A) Sampling was performed at 48 h and analyzed by real time-PCR (A) and zymography (B) for MMP-9, respectively. (B) EPO activity was measured in BALF 48 h after the last airway challenge. Data represent the means $\pm$ SEM from five independent experiments. * $p < 0.05$ and ** $p < 0.01$ vs. OVA-challenged mice.

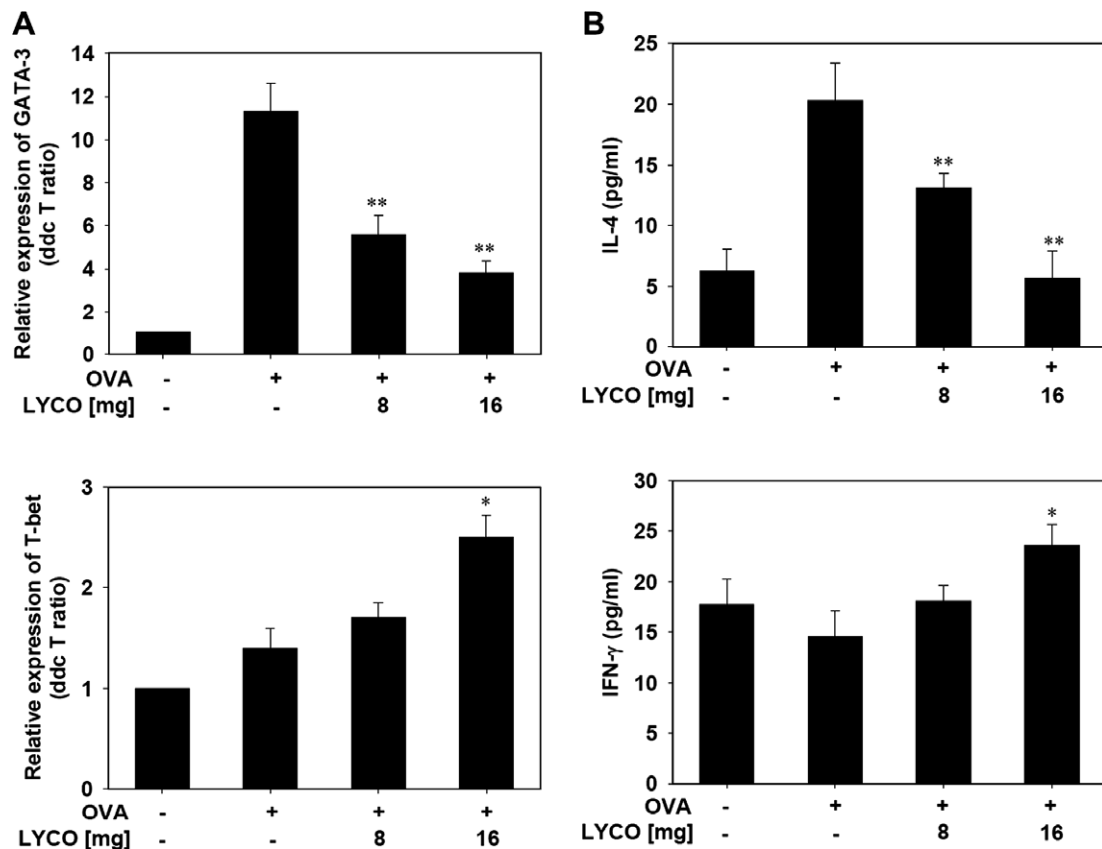


Fig. 4. Effect of lycopene (LYCO) on cytokine expression. Mice were sensitized and challenged by OVA as described in the Materials and methods. BAL was isolated at 4 h after the last challenge of OVA. Total RNA was isolated and real-time PCR was performed. GAPDH was used as an internal standard. (B) BAL fluids were collected 48 h after the last OVA challenge. IL-4 and IFN- γ levels were analyzed using ELISA ($n = 5$). Data represent the means $\pm$ SEM from five independent experiments. * $p < 0.05$ and ** $p < 0.01$ vs. OVA-challenged mice.

intraperitoneally the effect of lycopene on the expression and activity of MMP-9. These results suggest that the inhibition of

MMP-9 activity by lycopene blocks the infiltration of inflammatory cells into the lung.

It is well known that eosinophils are the most important effector cells in asthma [30]. The cells contain large amounts of granules that induce severe airway inflammation [31]. One of the inflammatory mediators, EPO, as a specific marker for eosinophil, has been shown increased levels in allergic patients [32]. Therefore, the inhibitory action of EPO is thought to be a good target for the treatment and prevention of asthma. Our results indicated that the lycopene inhibits EPO activity through suppressing the eosinophil influx into the airway and lung tissues.

The chronic asthma is well characterized that Th2 lymphocytes infiltrate into lung and produce inflammatory cytokines including IL-4, IL-5, and IL-13 [33]. These cytokines may exert the expression of inflammatory molecules both on the endothelial cells of the bronchial circulation and the airway epithelial cells, and may also promote recruitment of eosinophils and lymphocytes [34]. One of these, IL-4 especially induces isotype switching in B cells, from IgG to IgE production [35]. However, Th1 cytokines are known to inhibit allergic responses [36,37]. T-bet, a member of the T-box family of transcription factors, is a master determinant of Th1 lineage [38,39]. Indeed, T-bet deficient mice exhibit a profound lack of Th1 immune responses and ectopic expression of T-bet in murine directs activation of IFN- γ , as well as the upregulation of IL-12R β [40]. The expression of GATA-3 is, however, markedly upregulated in cells differentiating along the Th2 lineage, and is downregulated in cells differentiating along the Th1 pathway [41]. In this study, we observed that lycopene reduced the increased levels of IL-4 in OVA-challenged mice. However, it increased IFN- γ in lycopene administrated mice. Our data demonstrate that lycopene reduced the increased levels of GATA3 mRNA in OVA-challenged mice. It suggests that lycopene treatment is a novel, selective way to simultaneously suppress GATA-3 and increase T-bet expression in asthmatic reactions *in vivo* [40]. Taken together, these suggest that GATA-3 might be a regulator gene for asthma via Th1/Th2 balance.

In conclusion, our results strongly indicate that lycopene reduces allergic airway inflammation and asthma. Infiltration of inflammatory cells and expression of inflammatory mediators were attenuated by lycopene administration. Therefore, our data suggest that lycopene might offer a new therapeutic approach to allergic airway diseases.

Acknowledgments

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